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TITLE**: Cell Type-Specific Contributions to the TSC Neuropathology**

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Mutations in the human *TSC2* gene cause tuberous sclerosis complex (TSC), a developmental disorder characterized by tumor susceptibility and neurological manifestations. To better understand the disease we generated an animal model in which the mouse *Tsc2* gene is disrupted exclusively in excitatory neurons of the forebrain. We plan to investigate how heterozygous and homozygous *Tsc2* mutations affect the development of mutant excitatory neurons as well as other surrounding brain cells, in vivo and in vitro. During this first year of support we primarily focused on **Major Task 1:** *In vivo* characterization of heterozygous and homozygous NEX-*Tsc2* mice. Specifically, we performed Subtask 1: Animal breeding for *in vivo* studies, and nearly completed Subtask 3: Analysis of neuronal development and synaptogenesis. We studied the effects of *Tsc2* mutations on neuronal development and found that heterozygous loss of *Tsc2* in the forebrain has no effect, whereas homozygous loss disrupts the maturation of excitatory neurons.

15. SUBJECT TERMS
Tuberous Sclerosis Complex, animal model, TSC2

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Table of Contents

Page

1. Introduction

Mutations in the *TSC2* gene cause tuberous sclerosis complex (TSC), a developmental disorder characterized by tumor susceptibility in multiple organs and frequent neurological manifestations, such as seizures, intellectual disability, and autism. There is currently no cure for TSC, although significant progress has been made in recent years in managing some of the clinical traits, particularly tumor growth and epilepsy. However, much remains to be done to relieve the burden of epilepsy and cognitive problems, such as intellectual disability and autism. In line with the mission of TSCRP, the goal of this research is to improve our understanding of the pathogenesis of TSC, focusing on the cellular and molecular mechanisms that lead to the development of neurological symptoms. We believe that this basic knowledge can be translated into better treatments, and can ultimately improve the lives of TSC patients.

We hypothesized that heterozygous mutations in the *TSC2* gene disrupt the normal development and function of excitatory neurons without affecting their size. Homozygous loss-of-function mutations, on the other hand, not only profoundly alter their size and intrinsic development, but also disrupt cell-cell communication with other cell types. These non cell-autonomous mechanisms exacerbate defects in synaptic function and cognition, and possibly contribute to the formation of cortical tubers and tumors in the TSC brain.

2. Keywords

Tuberous Sclerosis Complex, animal model, TSC2

3. Accomplishments

- **Major goals of the project**

The overall goal of this study is to define the cellular abnormalities of excitatory neurons that are deficient in TSC2 activity, and to understand how they impact the development of other neuronal and glial subtypes in the cerebral cortex.

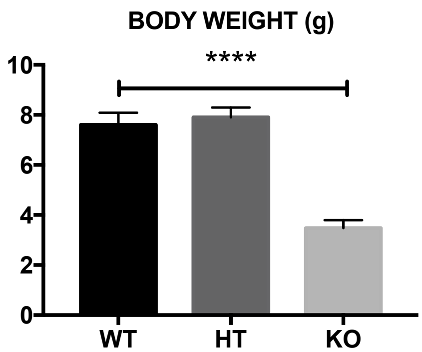
For our study we utilize NEX-*Tsc2,* a conditional *Tsc2* knock out mouse line in which gene deletion occurs specifically in embryonic forebrain excitatory neurons, the most abundant cell type in the developing cerebral cortex. We proposed to characterize the neurodevelopmental defects of heterozygous and homozygous NEX-*Tsc2* mice, including cell autonomous abnormalities in the migration and growth of excitatory neurons, as well as non-cell autonomous effects on inhibitory neurons and non-neuronal cell types. In collaboration with Dr. Anne Anderson, we also planned to use video-EEG recordings to determine whether heterozygous and homozygous NEX-*Tsc2* mice exhibit seizures or increased susceptibility to chemoconvulsants. Our proposed work includes in vivo studies (Specific Aim1), some of which were performed during this period, and in vitro culture studies (Specific Aim 2), which will be performed as planned in upcoming years of support.

As detailed in the SOW, during this initial period of support we focused on Major Task 1: *In vivo* characterization of heterozygous and homozygous NEX-*Tsc2* mice, corresponding to Specific Aim 1 of the research proposal.

- **Accomplishments under these goals**

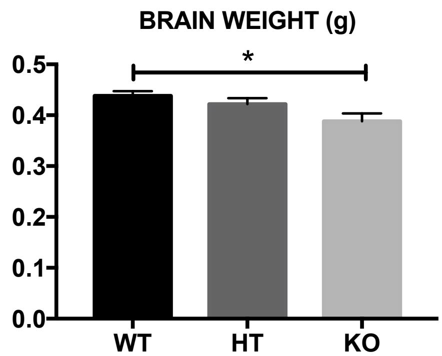
As per Subtask 1: Animal breeding for *in vivo* studies, we generated experimental NEX-Tsc2 mice by breeding $Cre^{+/+}$;$Tsc2^{flox/wt}$ mice with heterozygous mice $Cre^{-/-}$;$Tsc2^{flox/wt}$. This breeding strategy generated 100% $Cre^{+/-}$ mice that were either wild type (WT =

$Tsc2^{wt/wt}$), heterozygous (HET = $Tsc2^{fl/wt}$) or homozygous (KO = $Tsc2^{fl/fl}$) for *Tsc2*. Pups were born at the expected Mendelian ratio (25% WT, 50% HET, and 25% KO). As expected HET mice appeared indistinguishable from WT controls, whereas KO mutant mice appeared runt and some died prematurely at approximately 12-15 days after birth (P12-15). Despite some mortality, we were able to obtain 5 sets of P16 mice of each genotype (n= 5 WT, HET and KO) for our analysis. First, we measured the pups' body weight, and second, we euthanized them and dissected the brain to measure the weight of this organ. Statistical analysis was performed using the GraphPad Prism 7 software (one way ANOVA followed by Dunnett's multiple comparisons test). The data show that HET pups were similar to WT controls, but KO pups were significantly smaller. Similarly, the brain of HET pups was similar to that of WT controls, whereas the brain of KO pups was slightly but significantly smaller than WT.



WT vs. KO: p =0.0001

n= 5 mice/group



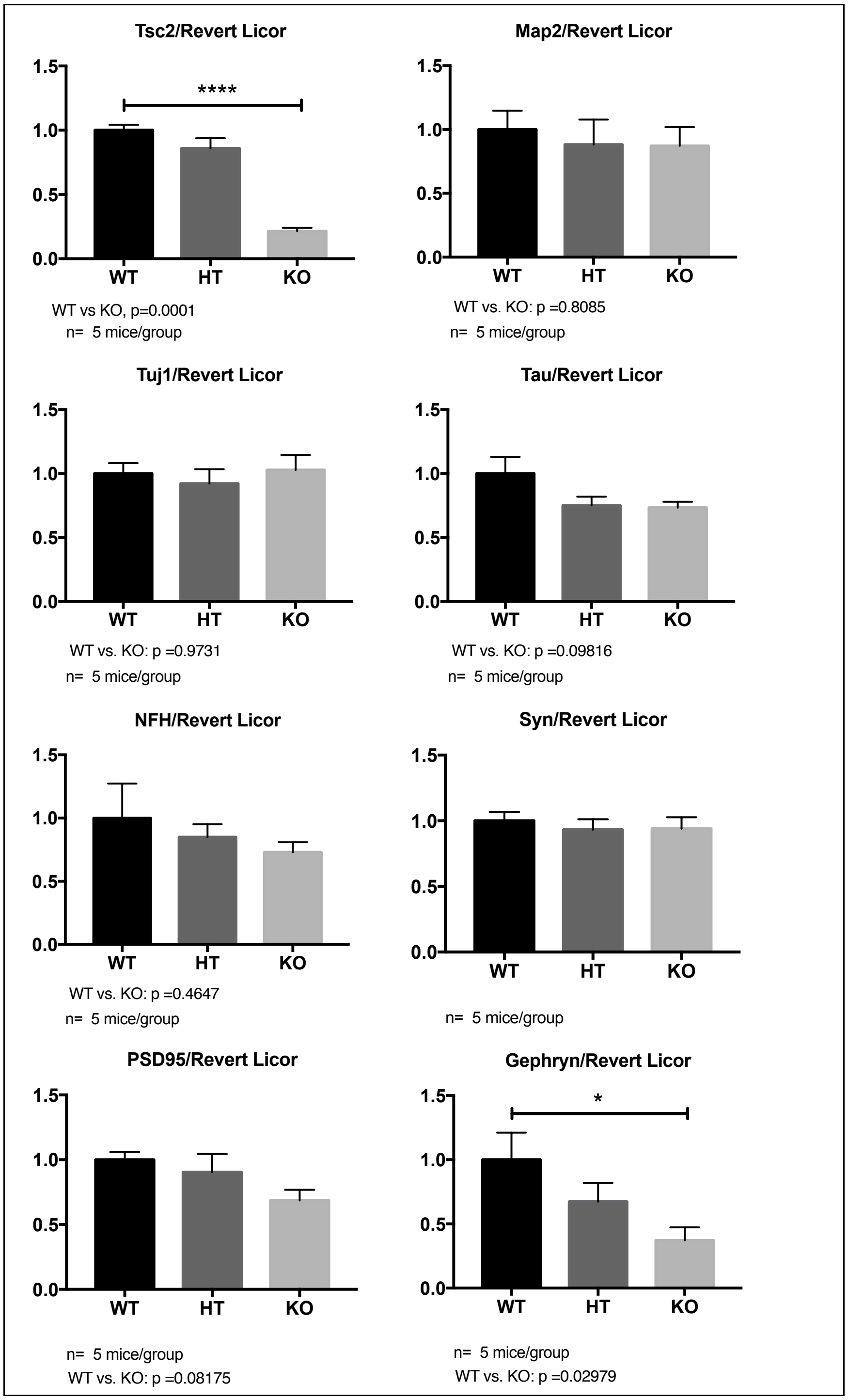
WT vs. KO: p =0.02674

n= 5 mice/group

To maintain the colony and regenerate breeder mice of the appropriate genotype we also interbred separately $Cre^{+/+}$;$Tsc2^{flox/wt}$ mice as well as $Cre^{-/-}$;$Tsc2^{flox/wt}$ and select $Tsc2^{flox/wt}$ from each progeny. These breeders were regenerated twice per year to ensure fertility.

As per Subtask 3: Analysis of neuronal development and synaptogenesis, we further dissected the cerebral cortex from P16 brains, prepared protein lysates and performed Western blot analysis using antibodies against axon, dendrite and synaptic markers. As a genotype control we analyzed the expression of Tsc2. Specific proteins were detected using the quantitative LI-COR Odyssey Fc imaging system. Blots will be stained with REVERT™ Total Protein Stain (Li-COR) to control for protein loading, followed by primary antibodies and secondary antibodies conjugated to near-infrared fluorophores (Li-COR). Statistical analysis was performed as described above.

The data plotted below show that there is no significant difference between genotypes regarding the expression levels of a dendrite marker (Map2), axon markers (TuJ1, Tau and NFH), a general presynaptic marker (Syn= synatophysin) or an excitatory postsynaptic marker (PSD95). However, there was a significant decrease in the expression of the inhibitory postsynaptic marker Gephryn in KO samples, and a marginal decrease in HET samples as well. These results suggest that Tsc2 deficiency in excitatory neurons results in a specific deficit in the formation of inhibitory synapses.



- **Opportunities for training and professional development**

Nothing to Report

- **Results disseminated**

Nothing to Report

- **Plan for next reporting period**

Next, we will perform Western blot analysis of synaptosomal fractions and immunofluorescence analysis of additional P16 NEX-Tsc2 mice as described in Subtask 3 to confirm our preliminary findings.

Furthermore, we will perform Subtask 2: Analysis of neuronal migration in the cerebral cortex, Subtask 4: Analysis of non-cell autonomous differentiation and signaling defects, Subtask 5: Analysis of non-cell autonomous effects on proliferation and cell death. Thus we will complete **Major Task 1:** *In vivo* characterization of heterozygous and homozygous NEX-*Tsc2* mice.

Depending on the novelty of the findings we may reach the first milestone and submit a *Manuscript on intrinsic neuronal abnormalities in Tsc2 mutants* for publication.

4. **Impact**

- **Impact on the development of the principal discipline of the project**

Our preliminary findings suggest that inhibitory synapses may be disrupted as a consequence of Tsc2 deficiency in NEX-Tsc2 HET and KO mice. If confirmed, this would be a novel and exciting result, pointing to a functional abnormality in synaptogenesis that may cause disinhibition of excitatory neurons in TSC patients as well as animal models. The deficit in the formation of inhibitory synapses could underlie the excessive neuronal activation and the seizures that are frequently associated with TSC.

- **Impact on other disciplines**

Nothing to Report

- **Impact on technology transfer**

Nothing to Report

- **impact on society**

Nothing to Report

5. **Changes/problems**

Nothing to Report

6. **Products**

Nothing to Report

7. **Participants & Other Collaborating Organizations**
    - **Individuals who worked on the project:**

Name	*Gabriella D'Arcangelo*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*darcangelo*
Nearest person month worked:	*3*

Contribution to Project:	*Dr. D'Arcangelo planned and supervised this study*
Funding Support:	*New Jersey State teaching line (Rutgers); this DOD award*
Name	*Beth Crowell*
Project Role:	*Senior lab technician*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*10*
Contribution to Project:	*Ms. Crowell managed the NEX-Tsc2 mouse colonies and performed most Western blot analysis*
Funding Support:	*This DOD award*
Name	*Avery Zucco*
Project Role:	*Graduate student*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*6*
Contribution to Project:	*Mr. Zucco helped performing some Western blot analysis*
Funding Support:	*Teaching assistant line; This DOD award*

- **Changes in active support**

Nothing to Report

- **Partners organizations**

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Since this is a collaborative award, an additional independent report will also be submitted by the Collaborating/Partnering PI (Dr. Anne Anderson, Baylor College of Medicine).

9. APPENDICES: None